

## AMENDMENTS TO THE SPECIFICATION

In the specification from page 8, line 29 to page 9, line 2, please delete the existing paragraph and replace with the following paragraph after implementing the following changes:

Figure 5 shows a comparison of NOS sequences. Phosphorylation site sequences for eNOS and nNOS are indicated in a schematic model of NOS. Sequences from the CaM-binding region (around the Thr-495 phosphorylation site in eNOS) and for the COOH-terminal tail (around the Ser-1177 phosphorylation site in eNOS) are shown. Top panel, human eNOS (SEQ ID NO:11); bovine eNOS (SEQ ID NO:12); human nNOS (SEQ ID NO:13); rat nNOS (SEQ ID NO:14); rat iNOS (SEQ ID NO:15); mouse iNOS (SEQ ID NO:16); and human iNOS (SEQ ID NO:17). Bottom panel, human eNOS (SEQ ID NO:18); bovine eNOS (SEQ ID NO:19); human nNOS (SEQ ID NO:20); rat nNOS (SEQ ID NO:21); rat iNOS (SEQ ID NO:22); mouse iNOS (SEQ ID NO:23); and human iNOS (SEQ ID NO:24).

In the specification at page 11, lines 15-20, please delete the existing paragraph and replace with the following paragraph after implementing the following changes:

Phosphopeptide isolation from in-gel tryptic digests was carried out as described by Mitchelhill et al (1997a). Greater than 98% of the radioactivity was recovered from the gel. Peptides isolated and characterized by mass spectrometry and Edman sequencing are set out in Table 1. KKTFKEVANAVK is SEQ ID NO:1; TQXFSLQER is SEQ ID NO:2; IRTQXFSLQER is SEQ ID NO:3; and pcLGSLVFPR is SEQ ID NO:4.

In the specification at page 13, lines 1-9, please delete the existing paragraph and replace with the following paragraph after implementing the following changes:

The location of the phosphorylation site in peptide A, TXFSLQER (SEQ ID NO:2), was identified by <sup>32</sup>P-phosphate release sequencing (Mitchelhill et al, 1997a). eNOS phosphorylated by the AMPK- $\alpha$ 1 was no longer recognized by the antibody to the eNOS COOH-terminal tail; nor was it eluted from the ADP-Sepharose affinity column by 100 mM NADPH. These properties prevented the direct confirmation of Ser-1177 phosphorylation *in situ*. This is illustrated in Venema et al, 1996.

In the specification at page 13, lines 10-18, please delete the existing paragraph and replace with the following paragraph after implementing the following changes:

A second site, Thr-495, was phosphorylated in the absence of Ca<sup>2+</sup>-CaM or when EGTA was present. This is illustrated in Figure 2 bottom panel, B. This residue is located in the CaM-binding sequence (SEQ ID NO:5),

TRKKT<sup>495</sup>FKEVANAVKISASLM,

between the oxidase and reductase domains of eNOS (Venema et al, 1996). Ser-101 in the N-terminal region of eNOS was identified as a minor site of phosphorylation (Figure 2 bottom panel, C).

In the specification at page 13, lines 19-31, please delete the existing paragraph and replace with the following paragraph after implementing the following changes:

Synthetic peptides containing Thr-495 or Ser-1177 were readily phosphorylated by AMPK, with similar kinetic values to the SAMS peptide substrate. The peptide containing Thr-495, GTGITRKKTFKEVANAVK (SEQ ID NO:6), was phosphorylated with a  $K_m$  of  $39 \pm 10 \mu M$  and a  $V_{max}$  of  $6.7 \pm 0.6 \mu mol/min/mg$ , whereas the peptide containing Ser-1177, RIRTQSFSLQERQLRG (SEQ ID NO:7) was phosphorylated with a  $K_m$  of  $54 \pm 6 \mu M$  and a  $V_{max}$  of  $5.8 \pm 0.3 \mu mol/min/mg$ . These are comparable to results obtained using the well-characterized SAMS peptide substrate, which has a  $K_m$   $33 \pm 3 \mu M$  and a  $V_{max}$  of  $8.1 \pm 1.5 \mu mol/min/mg$  (Michell et al, 1996). The *in vitro* phosphorylation of the peptides confirms the identification sites of phosphorylation.

In the specification at page 16, lines 6-16, please delete the existing paragraph and replace with the following paragraph after implementing the following changes:

Polyclonal antibodies were raised against synthetic phosphopeptides based on the eNOS sequence: RIRTQSpFSLQER (SEQ ID NO:8) and GITRKKTpFKEVANCV (SEQ ID NO:9). Rabbits were immunized with phosphopeptides coupled to keyhole limpet haemocyanin and then emulsified in Freund's complete adjuvant, using conventional methods. The antibodies were purified using the corresponding phosphopeptide affinity columns after thorough preclearing with dephosphopeptide affinity columns. The specificity of the purified antibodies was confirmed using both EIA and immunoblotting, confirming that they did not recognize recombinant dephospho-eNOS.

In the specification at page 18, lines 19-32, please delete the existing paragraph and replace with the following paragraph after implementing the following changes:

Since the identification of the Ser-1177 phosphorylation site by the present inventors, it has been recognized that other protein kinases phosphorylate at this site. In particular, the protein kinase Akt (also named PKB) phosphorylates Ser-1177 in response to stimulation of endothelial cells by vascular endothelial growth factor (VEGF) (Fulton et al. 1999; Michell et al., 1999) or to fluid shear stress (Dimmeler et al., 1999; Gallis et al., 1999). In the study by Gallis et al. (1999) it was reported that fluid shear stress stimulated the phosphorylation of Ser-116 in the sequence KLQTRPSPGPPPA (SEQ ID NO:10). Neither the kinase responsible nor the functional effects of phosphorylation of this site on eNOS has yet been identified. This phosphorylation site is present in the oxidase domain.

At the end of the specification, please insert the enclosed sequence listing.